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חוק הפטנטים, תשכ"ז-1967
Patent Law, 5727 - 1967

בקשה לפטנט
Application for Patent

לשימוש הלישכה
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אני, (שם המבקש, מענו ולגבי גוף מאוגד - מקום התאגדותו) I, (Name and address of applicant, and in case of body corporate-place of incorporation)

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ת.ד. 653
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(בעברית) (Hebrew)
גילוי קולורימטרי

(באנגלית) (English)
COLORIMETRIC DETECTION

hereby apply for a patent to be granted to me in respect thereof. מבקש בזאת כי ינתן לי עליה פטנט

*בקשת חלוקה - Application of Division	*בקשת פטנט מוסף - Application for Patent Addition	*דרישה דין קדימה Priority Claim		
*מבקשת פטנט from Application	*לבקשה/לפטנט to Patent/Appl.	מספר/סימן Number/Mark	תאריך Date	מדינת האיגוד Convention Country
מס' Nº _____	מס' Nº _____			
מיום dated _____	מיום dated _____			
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הוגש בעניין filed in case 126263				

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מספרנו: 7051/99

היום 14 בחודש מרץ שנה 1999
This of the year

חתימת המבקש
Signature of Applicant

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By:
Attorneys for Applicant

לשימוש הלישכה

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גילוי קולורימטרי

COLORIMETRIC DETECTION

Colorimetric Detection

Field of the Invention:

The present invention relates to the field of colorimetric detection of ions and peptides by mixed vesicles used therefor.

Background of the Invention

The use of ionophores for the selective detection of ions in aqueous solutions is described in the literature as are colorimetric methods. However the methods which utilize ionophores for detection of ions are generally based on complex mechanisms such as fluorescence and require analytical devices for detection and the use of various materials as carriers of the ionophores into the solution. Colorimetric methods known for detection of ions are generally based on chemical interactions between an indicator compound and the ion. Since ionophores are known to be hydrophobic many different methods of introducing them into aqueous solutions are known. The use of ionophores for colorimetric detection of ions, more particularly cations, combined with the use of specialized polydiacetylene (PDA) vesicles as carriers of ionophores into aqueous solutions are not known.

Many methods of evaluating peptides and their interaction with physiological membranes have been described in the literature. These methods are based on chemical and spectroscopic methods such as fluorescence, nuclear magnetic resonance and infra-red spectroscopy. Some of these methods have been known to utilize simple lipid based vesicles. The use of colorimetric detection of peptides and their activity and the use of PDA based matrix vesicles in the detection of peptides and peptide activity is not known.

It is therefore a purpose of the present invention to provide a highly selective colorimetric detection method of cations in solutions.

It is further a purpose of the present invention to provide novel carriers of ionophores into aqueous solutions.

It is yet a further purpose of the present invention to provide colorimetric detection of membrane-binding of short antibiotic peptides and membrane peptides.

A further purpose of the present invention is to provide a colorimetric detection method of membrane-binding of short antibiotic peptides and membrane peptides which utilizes a mixed vesicle assembly.

A further purpose of the present invention is to provide a detection colorimetric detection method which is sensitive to the amino acid sequence of the peptides wherein said peptides are peptides in which single amino acids have been replaced or omitted in positions which have structural and physiological importance.

Summary of the Invention

The present invention provides a selective colorimetric detection method using mixed vesicles comprising a polymerized matrix and phospholipids wherein supramolecular vesicle assemblies comprising of three constituents exhibit color transitions. Said method is used for the selective colorimetric detection method of specific cations in aqueous solutions characterized in using mixed vesicles comprising a polymerized PDA based matrix, phospholipids and ionophores. It has been discovered that supramolecular vesicle assemblies comprising of three constituents, namely polymerized polydiacetylene [PDA] matrix, phospholipids such as diinirystoylphosphatydilcholine [DMPC] and dipalmitoylphosphatydilcholine [DPPC], and ionophores, such as valinomycin and monensin, exhibit blue-to-red color transitions in the presence of small quantities of specific cations in solution. The colorimetric

transitions occur both when ions are added to a solution containing the mixed vesicles and the ionophores, or by adding ionophores into solutions containing the vesicles and the ions. Furthermore the present invention provides the use of said vesicles as ionophore carriers in aqueous solutions.

The present invention further provides colorimetric detection of membrane-binding of short antibiotic peptides and membrane peptides. Furthermore a colorimetric method which utilizes a vesicle assembly comprising of a polymerized PDA based matrix, such as polydiacetylene [PDA] matrix and phospholipid molecules, such as dimirystoylphosphatydilcholine [DMPC] and dipalmitoylphosphatydilcholine [DPPC] is provided. Color change is detected upon interactions of these vesicles with short antibiotic peptides, and with peptide domains which span physiological membranes wherein the color changes are not due to any chemical reaction or bond cleavage, but are characterized by physical interactions between membrane peptides and lipid bilayers wherein these physical interactions affect both the binding between the short peptides and phospholipid bilayers as well as conformational changes undergone by both the peptides and the lipid bilayers.

In a further aspect of the invention the detection is sensitive to the amino acid sequence of the peptides wherein said peptides are peptides in which single amino acids have been replaced or omitted in positions which have structural and physiological importance, give rise to different color changes compared with the native sequences.

Brief Description of the Drawings

Figure 1: Uv/vis absorption spectra of blue vesicles [intact PDA/DMPC vesicles, or PDA/DMPC/ionophore vesicles not in the presence of ions], and red vesicles [PDA/DMPC vesicles in the presence of melittin, or PDA/DMPC/ionophore in the presence of the selectively-bound cation].

Figure 2: Change of the ratio of absorbance at 500nm ["red" phase] and 620nm ["blue" phase] of DMPC/PDA vesicles mixed with monensin, upon addition of cation solutions at 50 μg/mL

Figure 3: Change of the ratio of absorbance at 500nm ["red" phase] and 620nm ["blue" phase] of DMPC/PDA vesicles mixed with valinomycin, upon addition of cation solutions at 50 μg/mL

Figure 4: Temporal change of relative absorbance of the peak at 620nm [blue], and at 500nm [red] after addition of melittin to: a. DMPC/PDA vesicles; and b. DPPC/PDA vesicles.

Figure 5: Relative intensity of peak at 500nm [red phase] observed for DMPC/PDA vesicles to which magainin and magainin analogs have been added, respectively.

Figure 6: Relative intensity of peak at 500nm [red phase] observed for DMPC/PDA vesicles to which melittin and melittin-L9W has been added.

Figure 7: Circular dichroism [CD] spectra of melittin in water solution; melittin in aqueous solution containing DMPC/PDA vesicles; melittin-L9W in aqueous solution containing DMPC/PDA vesicles.

Detailed Description of Preferred Embodiments of the Invention

The following description is illustrative of preferred embodiments of the invention. The following description is not to be construed as limiting, it being understood that the skilled person may carry out many obvious variations to the invention.

According to a preferred embodiment of the invention the colorimetric detection of cations in solution using mixed vesicles containing PDA/phospholipids/ionophores by supramolecular vesicle assemblies comprising of three constituents, namely polymerized polydiacetylene [PDA] matrix, phospholipids such as dimirystoylphosphatydilcholine [DMPC] and dipalmitoylphosphatydilcholine [DPPC], and ionophores, such as valinomycin and monensin, exhibit blue-to-red color transitions in the presence of small quantities of cations in solution. The colorimetric transitions occur both when ions are added to a solution containing the mixed vesicles and the ionophores, or by adding ionophores into solutions containing the vesicles and the ions. The color changes are dependent upon the ionophore embedded in the polymerized lipid matrix. In the presence of valinomycin, for example, the vesicles exhibit a blue-to-red color change upon addition of potassium ions [Figure 3], while in the case of DMPC/PDA vesicle mixture containing monensin there is a color change only in the presence of sodium ions [Figure 2]. Following the binding to the ions and the conformational changes of the ionophores, the vesicle structure is perturbed and a color change from blue to red is observed due to the perturbation. Because of the high selectivity of the ionophores, the vesicles will change color only in the presence of the particular ions binding to the respective ionophores in solution, making these molecular assemblies highly sensitive and selective ion-detection systems. Such ion sensitive systems are extremely important for many biological and medical-diagnostic applications. Potential applications include determination of extra- and intra-cellular cation concentrations, ion-channel activities, evaluation of the

effects of therapeutic drugs, which modify ion-channels, and general selective diagnostic kits for cations in solution.

According to a further preferred embodiment of the present invention a colorimetric detection method of membrane-binding of short antibiotic peptides and membrane peptides by utilizing a vesicle assembly consisting of a polydiacetylene [PDA] matrix and phospholipid molecules, such as dimirystoylphosphatydilcholine [DMPC] and dipalmitoylphosphatydilcholine [DPPC] is provided. Color change is detected upon interactions of these vesicles with short antibiotic peptides, and with peptide domains which span physiological membranes. The detection method is sensitive to the amino acid sequence of the peptides; peptides in which single amino acids have been replaced or omitted in positions which have structural and physiological importance, give rise to different color changes compared with the native sequences. Figure 1 depicts the uv-vis absorption spectra of the unperturbed DMPC/PDA vesicles [blue phase], and vesicles treated with 10μM solution of melittin [red phase]. The color changes are due to hydrophobic and electrostatic interactions between the phospholipids, which essentially form "membrane-like" domains within the PDA matrix, and the peptides. The color changes have been observed for solutions containing the peptides melittin, magainin, alamethicin, as well as transmembrane peptide domains such as M2 of the acetylcholine receptor. The color changes are not due to any chemical reaction or bond cleavage, but rather to the physical interactions between membrane peptides and lipid bilayers. These physical interactions affect both the binding between the short peptides and phospholipid bilayers as well as conformational changes undergone by both the peptides and the lipid bilayers. One evidence for the above is that the blue-to-red color changes of mixed phospholipid/PDA vesicles in the presence of melittin, for example, occur at room temperature only in the presence of DMPC, which forms a fluid lipid phase at this temperature.

However, when the PDA vesicles contain DPPC – which is in the gel-phase at room temperature, no color change is observed since melittin do not penetrate lipid bilayers in the gel phase. These results are shown in Figure 4. The present invention is sensitive to the amino acid sequence of the peptides; peptides in which single amino acids have been replaced or omitted in position, which have structural and physiological importance, give rise to different color changes compared with the native sequences. The differences in color changes arise from two related mechanisms. In one group of antimicrobial peptide analogs, there are no or minimal interactions of the analogs with the membrane domains in the PDA matrix thus producing no color change. This is shown for magainin and its analogs in Figure 5. Indeed Figure 5 shows insignificant color changes induced by magainin-analogs in which lysine-11 has been replaced with glutamic acid, and in the sequence where both lysine-10 and lysine-11 have been similarly replaced with glutamic acid. In both cases the replacement of the positively-charged residues significantly reduces the electrostatic driving force for magainin to bind to the phosphate moieties with the phospholipids.

In another group of peptide analogs examined, alteration of certain amino-acids gives rise to drastically different chromatic effects, an example of which is depicted in Figure 6. In several melittin analogs in which amino acids have been altered or omitted in the putative helix regions of the peptide we have detected enhanced colorimetric responses of DMPC/PDA vesicles, as compared with the native melittin sequence. Figure 6 depicts, for example, a comparison between the extent of appearance of red color of the vesicles following interaction with native melittin and a melittin analog in which Leu-9 has been replaced with tryptophan (melittin-L9W), respectively. The vesicles appear clearly more reddish upon interaction with melittin-L9W compared with the native sequence. Furthermore, we find that the color changes associated with several melittin analogs examined occur faster than native melittin, and are often accompanied by aggregation

and precipitation of the vesicles. These observations most likely indicate that the melittin analogs adsorb onto the head-groups of the phospholipids through electrostatic interactions with the charged residues in the sequence. However unlike native melittin, the analogs do not penetrate into the lipid bilayer because their helix-formation capabilities have been reduced or eliminated. Indeed, the leucine residue at position 9 in melittin is critical for the formation of an alpha-helix. Following the electrostatic interactions with the phosphate headgroups of the phospholipids, the melittin analogs induce higher surface perturbation of the vesicles, thus giving rise to the observed enhanced color changes. This analysis is supported by circular dichroism [CD] data shown in Figure 7. The CD spectrum shows that melittin clearly adopts a helical conformation in the presence of DMPC/PDA vesicles, indicated by the minimum observed at around 208nm. However, the melittin analog in which Leu-9 has been replaced with tryptophan does not form a helix, but rather gives rise to CD spectra associated with a sheet-like structure. Because of the direct relationships between the peptide sequences and color change of the vesicles, the technique, which we have discovered, may have important uses in biopharmaceutical applications and biochemical research-and-development. Among the potential applications are: high-throughout screening of antibiotic peptides libraries, diagnostic kits for antibiotic peptides, development of anti-microbial drugs which induce membrane perturbations and/or lysis, and elucidating the factors affecting peptide-membrane interactions.

CLAIMS

1. A selective colorimetric detection method using mixed vesicles comprising a polymerized matrix and phospholipids wherein supramolecular vesicle assemblies comprising of three constituents exhibit color transitions.

2. A method according to claim 1 for the detection of cations in aqueous solutions.

3. A method according to claim 2 wherein the vesicle further comprises an ionophore.

4. A method according to claim 3 wherein supramolecular vesicle assemblies comprising of three constituents exhibit blue-to-red color transitions in the presence of small quantities of cations in aqueous solutions.

5. A method according to claim 1 wherein the polymerized matrix of the vesicle is a polymerized polydiacetylene [PDA] matrix.

6. A method according to claim 1 wherein the phospholipid is selected from dimirystoylphosphatydilcholine [DMPC] and dipalmitoylphosphatydilcholine [DPPC].

7. A method according to claim 1 wherein the ionophores are selected from among valinomycin and monensin.

8. A method according to claim 4 wherein the colorimetric transitions occur when ions are added to a solution containing the vesicles and the ions.

9. A method according to claim 4 wherein the colorimetric transitions occurs by adding ionophores into solutions containing the vesicles and the ions.

10. A vesicle as described in claims 5 and 6 for use as means for introducing ionophores into aqueous solutions.

11. A method according to claim 1 for colorimetric detection of membrane-binding of short antibiotic peptides and membrane peptides.

12. Colorimetric detection according to claim 11 utilizing a vesicle assembly comprising of a polymerized matrix and phospholipid molecules, wherein the color change is detected upon interactions of these vesicles with short antibiotic peptides, and with peptide domains which span physiological membranes characterized in that the color changes are due to physical interactions between membrane peptides and lipid bilayers.

13. Colorimetric detection according to claim 12 wherein the polymerized matrix is polydiacetylene [PDA] matrix.

14. Colorimetric detection according to claim 11 wherein the phospholipid molecule is selected from among dimirystoylphosphatydilcholine [DMPC] and dipalmitoylphosphatydilcholine [DPPC].

15. Colorimetric detection according to claim 12 wherein said physical interactions affect both the binding between the short peptides and phospholipid bilayers as well as conformational changes undergone by both the peptides and the lipid bilayers.

16. Colorimetric detection according to claim 11 which is sensitive to the amino acid sequence of the peptides detected.

17. Peptides as described in claim 16 in which single amino acids have been replaced or omitted in positions which have structural and physiological importance, give rise to different color changes compared with the native sequences.

לוצאטו את לוצאטו
LUZZATTO & LUZZATTO
By ע"י

Figure 1

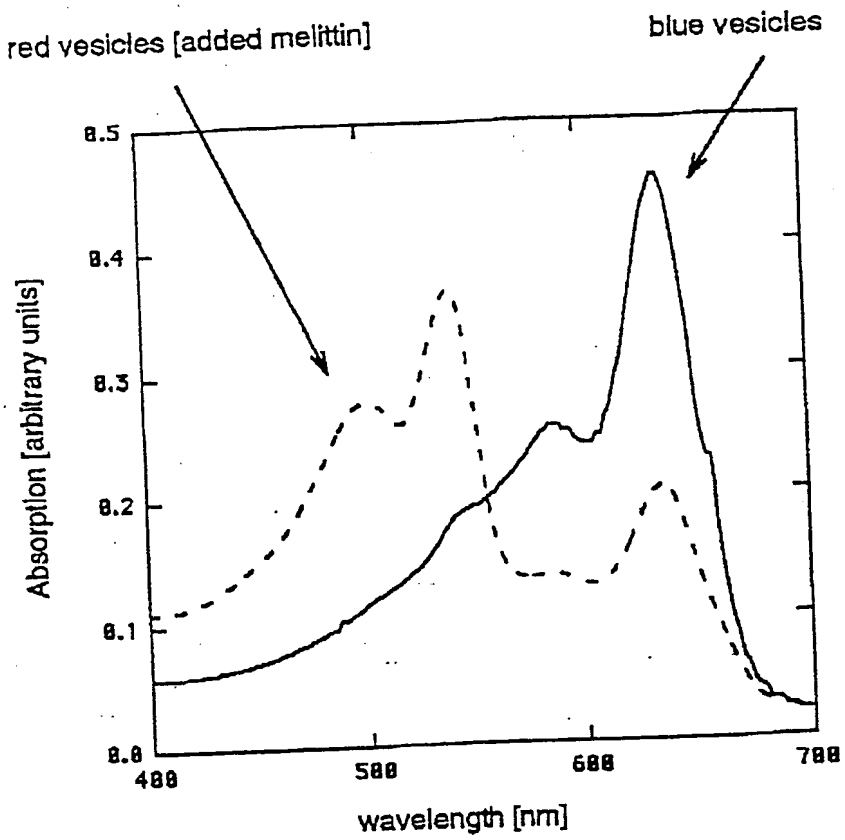

red vesicles [added melittin]
blue vesicles
Absorption [arbitrary units]
0.5
0.4
0.3
0.2
0.1
0.0
400
500
600
700
wavelength [nm]

Figure 2: monensin

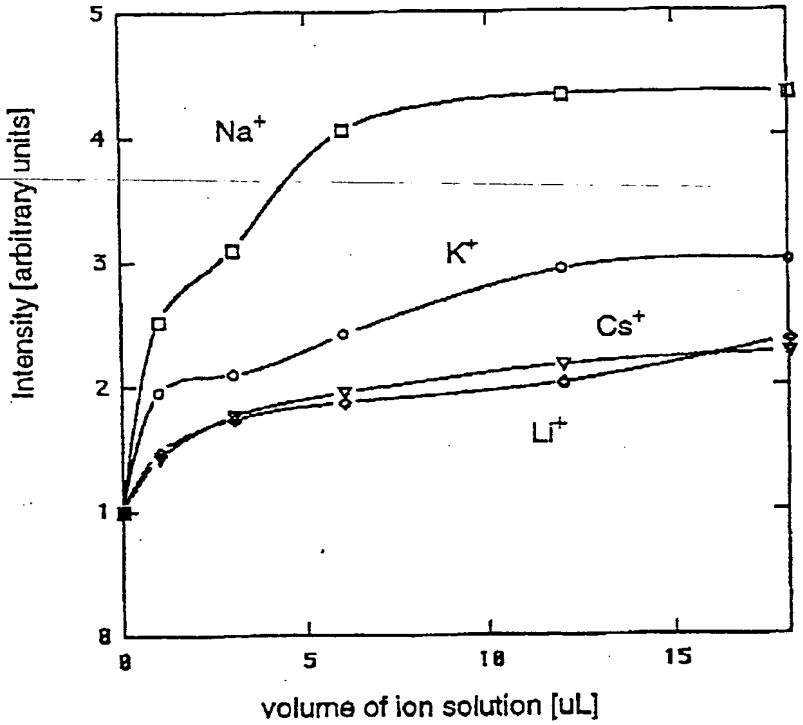


Figure 3: valinomycin

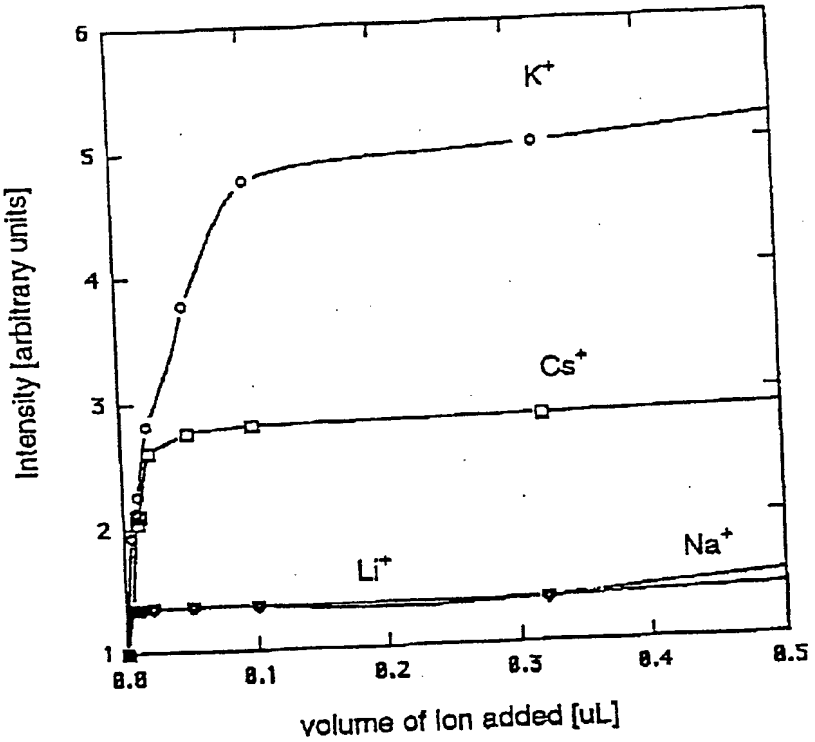
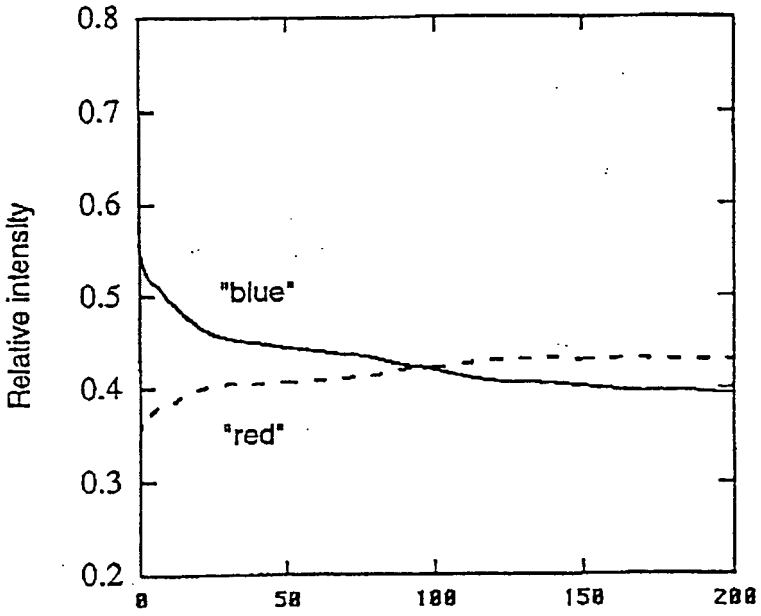


Figure 4

a. melittin + DMPC/PDA vesicles



b. melittin + DPPC/PDA vesicles

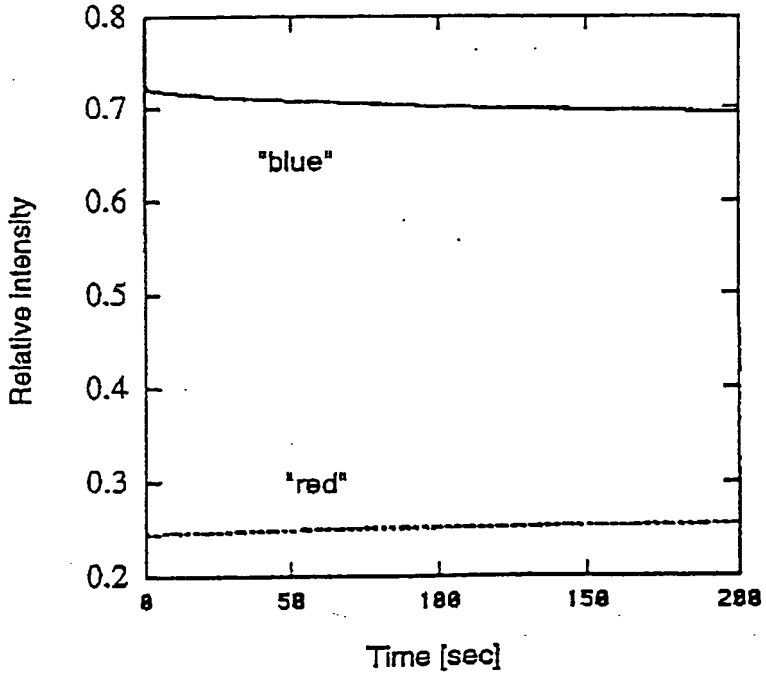


Figure 5

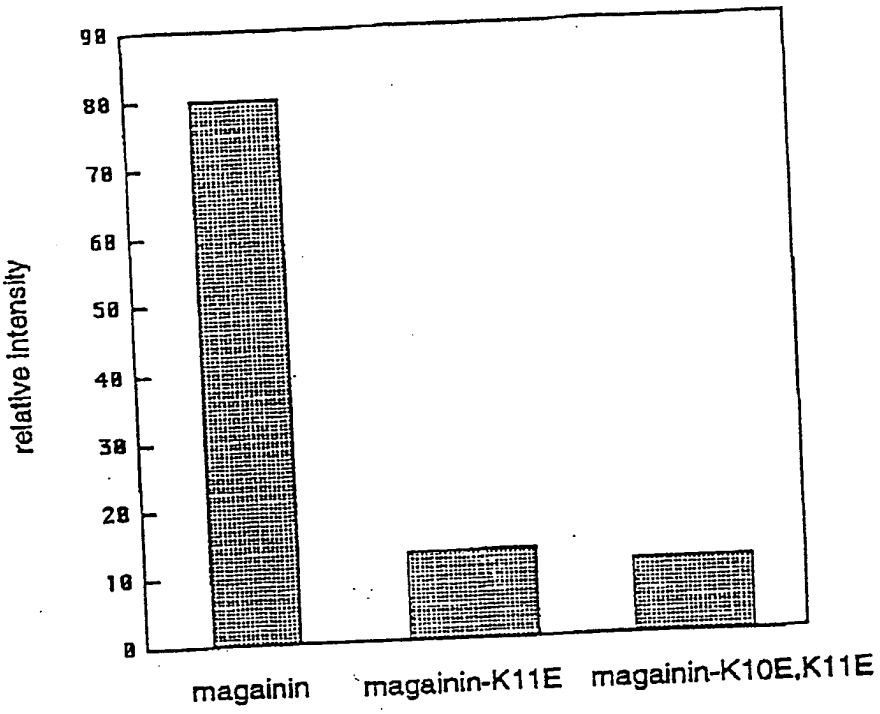

relative intensity
90
80
70
60
50
40
30
20
10
0
magainin
magainin-K11E
magainin-K10E,K11E

Figure 6

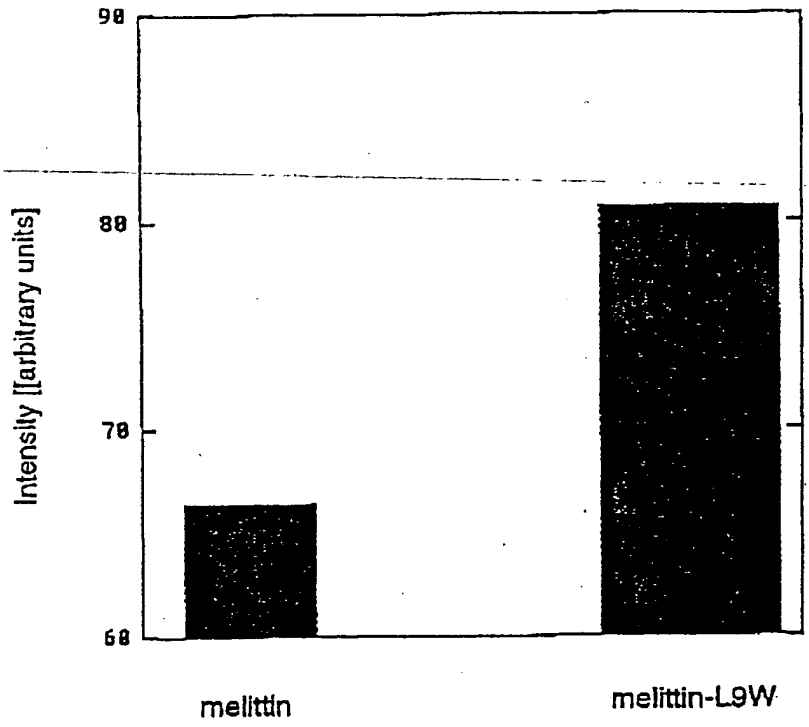
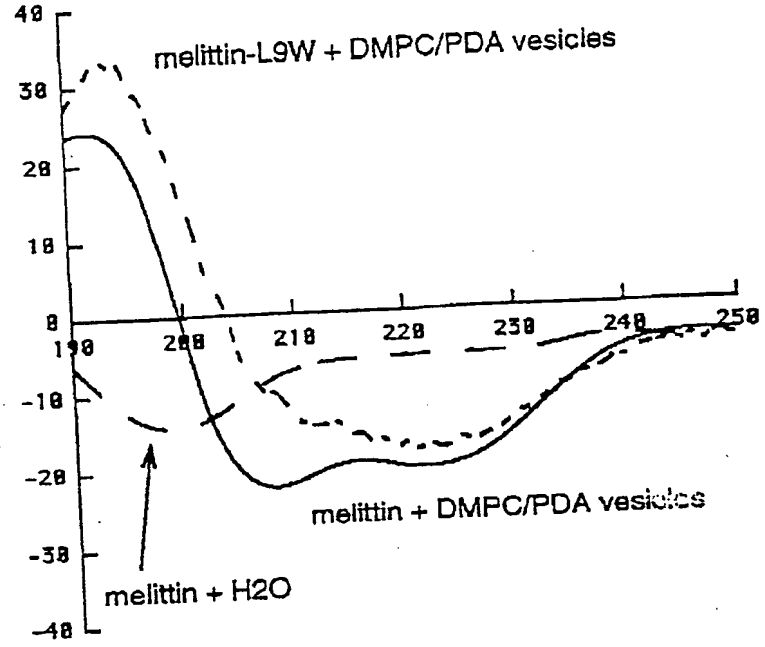

90
80
70
60
Intensity [[arbitrary units]
melittin
melittin-L9W

Figure: 7


40
30
20
10
0
-10
-20
-30
-40
190
200
210
220
230
240
250
melittin-L9W + DMPC/PDA vesicles
melittin + DMPC/PDA vesicles
melittin + H2O